

**Is there an association between obstructive sleep apnea syndrome and periodontal inflammation? Evaluation of biochemical and microbiological findings.**

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## **Abstract**

**Objectives:** To assess salivary, serum biomarkers, and subgingival bacteria as putative candidates in the potential association between obstructive sleep apnea syndrome (OSAS) and periodontal disease.

**Materials and methods:** Fifty-two patients were grouped according to the severity of OSAS: 13 participants served as controls, 17 patients had mild-to-moderate OSAS, and 22 severe OSAS. Serum, saliva, and subgingival plaque samples were collected, clinical periodontal parameters recorded. Salivary, serum concentrations of interleukin-6 (IL-6), tumour necrosis factor (TNF- $\alpha$ ), osteoprotegerin, sRANKL, and apelin were analysed by enzyme-linked immunosorbent assay. Bacterial counts were determined by real-time QPCR on plaque microbial DNA preparations.

**Results:** There was a significant change in the composition of microbes in plaque particularly in severe OSAS samples ( $p < 0.01$ ). Statistical analyses indicated significantly higher salivary IL-6 levels in both OSAS groups compared to controls ( $p < 0.05$ ). Salivary apelin levels were significantly higher in Serum levels of these biomarkers and salivary osteoprotegerin, sRANKL levels were similar in the study groups. The incidence and duration of apnea positively correlated with clinical periodontal parameters ( $p < 0.05$ ).

**Conclusion:** OSAS appeared to alter the tested bacteria in plaque, correlate with increasing periodontal disease severity, have additive effect on salivary IL-6.

**Clinical relevance:** OSAS is likely to interact with periodontal disease.

**Key words:** Apelin; interleukin-6; osteoprotegerin; plaque; obstructive sleep apnea; periodontal disease.

## Introduction

Obstructive sleep apnea syndrome (OSAS) is characterized by recurrent events of upper airway obstruction during sleep associated with accompanying clinical signs and related symptoms [1]. OSAS is clinically characterized by repetitive phases of partial (hypopnea) or complete (apnea) upper airflow obstruction. This leads to a lowering of haemoglobin oxygen saturation and sleep cycle fragmentation [2]. Systemic inflammation and increased circulating levels of inflammatory markers have been also associated with OSAS [3]. The apnea-hypopnea index (AHI) is the total number of apneas and hypopneas per hour during sleep, and it is used to determine both the presence and the severity of OSAS [4]. OSAS is indicated when the AHI score is  $\geq 5$  with accompanying clinical symptoms such as excessive daytime sleepiness, loud snoring and nocturnal choking or when the AHI score is  $\geq 15$  without clinical symptoms [5]. An AHI score  $< 5$  is accepted within normal limits (non-OSAS), and scores between 5-15, 15-30, and  $\geq 30$  are indicative of mild, moderate, and severe OSAS, respectively [4,5].

Interleukin-6 (IL-6) is a multifunctional cytokine that has several biological activities, such as T-lymphocyte proliferation, B-lymphocyte differentiation and the stimulation of immunoglobulin secretion [6]. Notably, IL-6 activates bone resorption alone and in conjunction with other bone resorbing agents [7]. Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a potent inflammatory cytokine, which up regulates the production of prostaglandin, collagenases, cytokines, cell adhesion molecules and factors related in bone resorption [8,9]. Receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG), members of TNF and TNF receptor superfamily, are also known to play important roles in osteoclastogenesis [10]. RANKL first binds RANK and then the surface receptors of osteoclasts and preosteoclasts, activates the mature osteoclasts and sets up the differentiation of osteoclast progenitors [11]. Besides, OPG is a decoy receptor

for RANKL, and also a competitive glycoprotein inhibitor of osteoclast differentiation and activity [12]. IL-6 was found to be higher in plasma samples of OSAS patients compared to non-OSAS individuals [3]. IL-6 was also higher in the gingival crevicular fluid (GCF) samples of periodontitis patients as it was for TNF- $\alpha$  levels [8,13]. Apelin is a peptide hormone, which is thought to have an important role in respiratory physiology [14]. Untreated obstructive sleep apnea was associated with elevated plasma apelin levels, which restored after continuous positive airway pressure (CPAP) therapy [15].

Higher prevalence of periodontitis was reported in patients with OSAS [16] and major risk factors for periodontitis such as old age, male gender, smoking, alcohol consumption, diabetes and obesity [17-19] are generally common in OSAS [20]. Furthermore, both periodontitis and OSAS are associated with systemic inflammation possibly involving similar pathways and have been suggested to affect development of systemic diseases such as cardiovascular disease [21] and diabetes [22]. Increased levels of inflammatory markers have been proposed to have a role in periodontal disease and on interactions with systemic diseases [23,24].

It is hypothesized that OSAS may be associated with saliva, serum levels of biomarkers, subgingival bacteria counts and clinical periodontal status. Therefore, the aim of the present study was to compare saliva, serum IL-6, TNF- $\alpha$ , OPG, sRANKL and apelin concentrations and subgingival bacteria counts together with clinical periodontal status in patients with or without OSAS.

## **Materials and Methods**

### **Study Population**

Although 159 subjects were screened for inclusion into the study a total of 107 subjects were excluded because they did not meet the inclusion criteria, did not wish to participate

in the study or for undisclosed reasons (Figure 1). Fifty-two patients (20 females and 32 males; with an age range of 21 to 64 years) seeking treatment due to sleep apnea-related symptoms at the Department of Chest Disease, School of Medicine, Ege University were recruited for the study between January 2011 and September 2012

Seventeen patients (8 females and 9 males; age range 29 to 64 years) were diagnosed to have mild-to-moderate OSAS and 22 patients (4 females and 18 males; age range 26 to 61 years) in the severe OSAS group. Thirteen individuals (8 females and 5 males; age range 21 to 59 years) were included in the non-OSAS control group.

Smoking status, alcohol and psychotropic drug use, and anthropometric measurements such as body mass index, circumferences of neck, waist and hip, and medical histories were carefully evaluated. Pulmonary function tests and blood gas analysis were also performed in all subjects. Patients who had diabetes mellitus, immunological disorders, arthritis/osteoporosis, history of periodontal intervention within the last 6 months or antibiotic treatment within the last 3 months were excluded from the study. Eligible individuals had  $\geq 20$  teeth and were not using removable partial dentures. Smoking status of the patients was categorized as current-smoker, former smoker and non-smoker. Patients who smoke  $\geq 10$  cigarettes/day for  $> 5$  years were current-smokers and those who smoke  $< 10$  cigarettes/day for  $< 5$  years and had quit smoking at least 6 months prior to enrolment in the study were the former smokers [25]. Non-smokers had no history of smoking.

The study was approved by the Ethics Committee of Ege University, School of Medicine with the protocol number 15-1.1/10, and conducted in full accordance with ethical principles, including the World Medical Association's Declaration of Helsinki, as revised in 2002. The study protocol was thoroughly explained to all individuals, and written informed consent was received from each individual before inclusion in the study.

### **Measurement of Clinical Periodontal Parameters**

Following collection of saliva and serum samples, clinical periodontal parameters were measured and recorded by a single examiner (NN) blinded to the sleep apnea status of the patients. Before the initiation of the study, intra-examiner calibration was tested and validated by an intraclass correlation coefficient of 0.90 for both probing depth (PD) and clinical attachment level (CAL) measures. Dichotomous plaque index (PI) [26] (as present or absent), PD, and bleeding on probing (BOP; as present or absent within 15 seconds after periodontal probing) were recorded at 6 sites using a periodontal probe (Williams periodontal probe, Hu-Friedy, Chicago, IL, USA ) on each tooth present, except the third molars.

### **Saliva and Serum Sampling**

Whole saliva samples were collected as previously described by Navazesh [27] simply by expectorating into polypropylene tubes, in the morning following an overnight fast during which patients were asked not to chew gum or drink anything except water. 500  $\mu$ L of saliva samples were placed in polypropylene tubes following clarification by centrifugation (800 g) for 10 min.

The serum was separated by centrifugation of 5 mL venous blood sample taken from the antecubital vein by a standard venipuncture method. Centrifugation was made at 1500 g for 10 min and 500  $\mu$ L of aliquots were placed in Eppendorf tubes. Both serum and saliva samples were immediately frozen and stored at  $-80^{\circ}\text{C}$  and thawed immediately before the biochemical analyses.

### **Subgingival Plaque Sampling**

Subgingival plaque samples were collected as described previously [28] from the two

deepest pockets of the dentition. Prior to sampling, the sample teeth were isolated with cotton rolls and the sites were cleaned of saliva and supragingival plaque using sterile cotton pellets. A sterile paperpoint was quickly inserted to the base of the periodontal pocket, left there for 10 s and then removed gently. Maximum care was taken not to provoke any bleeding in the adjacent tissues. The two pocket samples were pooled in dry Eppendorf tube and the samples were kept at -80°C until the microbiological analyses were performed.

### **Biochemical Analyses**

ELISA or EIA kits were purchased for sRANKL (soluble, uncomplexed RANKL) (Peprotech, London, UK), OPG and IL-6 (R & D systems, Abingdon UK), TNF- $\alpha$  (Invitrogen, Paisley, UK) and Apelin kit which targets the C-terminus of the 77 amino acid peptide and detects all apelin peptides (Sigma, Poole UK). The ELISA assays were carried out according to the manufacturers' recommendations. The lower limits of detection for each assay were 1.6 pg/mL for TNF- $\alpha$ ; 3.2 pg/mL for IL-6; 125 pg/mL for sRANKL; 250pg/mL for OPG; and 2.5 pg/mL for apelin.

### **Microbiological Analyses**

To provide DNA from known amounts of each test species for use as standards in the Real-time quantitative polymerase chain reaction (QPCR) analysis of plaque samples, bacterial type strains were grown on agar plates prepared with the appropriate culture media, and atmospheric conditions for 24-72 hours (dependent on the microbe). A single colony was then picked and this was grown in liquid culture under the appropriate conditions for 24 h. An aliquot was taken to determine the colony forming units.

### **Preparation and Assessment of Genomic DNA**

The Epicentre Masterpure Gram positive DNA isolation kit (Cambio, Cambridge, UK) was used to prepare genomic DNA from the test strains (see above) and oral plaque sample bacteria from the paper points. Paper points were vortexed for 30 seconds in the TE buffer and the manufacturer's protocol followed. The amount of DNA and the purity was checked at 260nm and 280 nm (260/280nm ratio = 1.8–2.0 =good DNA purity) using a NANODROP 1000 spectrophotometer (Thermo Renfrew, UK). The DNA in standard samples was measured by fluorimetric analysis using the CYquant assay system (Invitrogen Paisley, UK) and the value plotted against the number of CFU/ml obtained in the original culture and these concentrations were adjusted to use as standards for each bacterial assay.

### **Real-time quantitative polymerase chain reaction**

A RT-QPCR assay using either TaqMAN or SYBRgreen chemistry (ABI/Invitrogen Paisley UK) was used for the detection and quantification of bacterial cell numbers/ CFU equivalents in the Real time QPCR. The primers and probes selected for the following bacteria were as published in the sources shown: *P.gingivalis* *A. actinomycetemcomitans*, *P. intermedia*, *P. micra*, *T. forsythia*, *F. nucleatum* [29-32] *T. denticola* [33], *S. mutans*, *F. nucleatum*, *S. oralis*, *A. naeshundi* [34], *V. dispar* [35] and were purchased from Invitrogen (Paisley, UK) or Eurogentech (Liege, Belgium).

Statagene MRX III thermal cycler (Agilent, Edinburgh, UK), the TaqMAN assay PCR cycling parameters used in the study were 10 min at 95°C, and 40 cycles of 30 s at 95°C and 1 min at 60°C. The cycles with SYBRgreen were as follows 10 min at 95°C, and 40 cycles of 30 s at 95°C and 1 min at 60°C and 1 min at 72°C (used for *A. naeshundi* and *V. dispar*). All primer sets were validated by running four serial 1/10 dilutions of the standard DNA and calculating the efficiency of the reaction (E) where

$$E = (10^{-1/\text{slope}}) - 1.$$



All reaction efficiencies calculated were acceptable (between 91% and 104%). All primer sets failed to amplify the DNA purified from different microbial standards.

## **Statistical Analyses**

Microsoft Excel and a commercially available statistical software program (SPSS Inc. version 21 IBM, Chicago, USA) was used to analyse the data. After assessing the data distribution for each parameter on a Q-Q plot, natural log data transformations were performed where necessary. Statistical power calculation was based on ANOVA where  $\alpha=0.5$ , 80% statistical power was attainable when the number in each group corresponds to a predicted effect size ( $n=33$  the effect size  $=0.8$ ,  $n=21$  the effect size  $=1$  and  $n=13$  the effect size  $=1.3$ ). The one-way ANOVA test was performed on the data and used to evaluate the differences between groups. Depending on the significance of a Levene's test, a Games-Howell or Tukey test served as the *post-hoc* test. Chi square and Univariate tests were utilized to determine potential confounding factors in the analysis. Weighted least squares regression analysis using ANOVA and appropriate post tests, were performed in a general linear model to correct for potential confounding factors including participant gender and periodontal disease for example. The differences in bacteria detected in the participant groups were investigated by a principal component analysis and summation plot of CFU or proportion of total collected.

Correlation between the clinical parameters and the microbial analysis, and between salivary and serum biochemical data were evaluated with Spearman rho rank correlation test. All tests were performed at  $\alpha = 0.05$  significance level.

## **Results**

There was no significant difference in the clinical and demographic parameters between the study groups apart from the gender ratios and AHI scores (Table 1). The male/female ratio was higher in the severe OSAS group than the mild-to-moderate OSAS and control groups ( $p<0.05$ ) (Table 1). As potential confounding factors, gender and AHI score were compensated for in a weighted analysis of the clinical and biochemical data.

There was a distinct change in the composition of the microbial plaque according to the principal component analysis in OSAS patients (Fig.2). All of the selected bacteria: *A.actinomycescomitans*, *A. naeslundii*, *F.nucleatum*, *P. gingivalis*, *P.intermedia*, *P. micra*, *S.mutans*, *S. oralis*, *T.denticola*, *T. forsythia*, and *V.dispar* were found in the patient groups, but not in every subject (Table 2) within those groups. The total mean CFU equivalent counts adjusted to the DNA concentration increased according to OSAS severity (Fig. 3A) and the proportion each bacterium tested of the total detected (Fig. 3B) was also altered significantly ( $p<0.01$ ). There was a fall in the number of the test microbes found in the mild to moderately affected OSAS patients when compared with the control group, but no significant difference was seen in the types of organisms detected when compared with the control group.

Marked increases in Gram negative bacteria tested in plaque samples of the most severely affected patients with OSAS and periodontal disease were seen. An increase in the Gram positive bacteria tested was also seen in plaque samples in the severe OSAS cases (Fig. 3A).

Salivary IL-6 levels were significantly higher in the OSAS groups compared to the non-OSAS control group (Table 2). There was no statistically significant difference in salivary IL-6 levels between the two OSAS groups ( $p<0.05$ ). Salivary apelin levels were significantly higher in the severe OSAS group compared to the control group. The levels of salivary TNF- $\alpha$ , sRANKL, OPG and OPG/sRANKL ratio were all similar in the study

groups (Table 3). Serum IL-6 and apelin concentrations were higher in the OSAS groups, but there was no statistically significant difference in the serum biomarkers between the study groups (Table 3).

The only significant correlation between salivary and serum biomarkers was found in IL-6 levels ( $\rho = 0.311$ ;  $p = 0.038$ ). Salivary IL-6 correlated significantly also with predictors of OSAS severity (AHI, oxygen desaturation index).

The mean length and the longest duration of sleep apnea, the duration of apnea-hypopnea correlated with PD, CAL and the number of episodes of apnea correlated with CAL (Table 4).

## **Discussion**

Despite our intention to recruit more participants to the study, the low number of participants due to the high frequency of various systemic diseases in patients with OSAS and failure of many potential participants fulfilling the systemic and/or oral inclusion criteria is recognised as a major limitation in our study. This also deterred the formation of subgroups based on the periodontal status (healthy-gingivitis-chronic periodontitis) among the OSAS categories.

Within the limits of this case-control study, we were able to detect greater numbers of periodontally relevant pathogenic micro-organisms in patients with the most severe OSAS and these people appeared to show the greatest shift in the composition of the microbes detected, which appears to support our hypothesis that OSAS impacts upon the colonisation and potential infection of the oral mucosa. A limitation of the QPCR approach is that there is a large proportion of the microbial community remains unaccounted for. This may in part explain why we see an apparent fall in the number of bacteria detected in Mild to moderate OSAS while the total DNA content remained similar. However, it is well documented that the microbial diversity is usually lower in the

periodontal disease state than in health [36]. The numbers of periodontally relevant pathogens were greater in the patients with severe OSAS, that they made up a significantly greater proportion of the oral microbiome than in the people with no OSAS or mild/moderate OSAS. This finding makes it tempting to suggest that our results support the idea that the diversity of microbes is reduced in severe OSAS. However, further analysis of the oral microbiome in such individuals is required to prove this hypothesis.

The influence of OSAS on bacterial colonization and periodontal disease may be explained by a low-grade inflammation associated with OSAS together with intermittent decrease in oxygenation. These features may contribute to differences in microbial ecology, which might influence onset and/or progression of periodontitis. Drying of the oral cavity due to mouth breathing in OSAS may also prevent self-cleaning ability of the oral mucosa and result in increased bacterial colonization [37]. A higher prevalence of periodontitis has been reported in patients with OSAS [1] and both diseases share the same risk factors [17-19]. OSAS and periodontitis are also known to affect similar systemic diseases such as cardiovascular diseases and diabetes [21,22]. Systemic effects on the same diseases might be explained with similar inflammatory pathways, which can also be the link between these two chronic diseases. Obesity is another common risk factor to be considered in this regard and it has a significant association with periodontitis via mechanisms associated with BMI, body fat, and maximum oxygen consumption [38]. In the present study, the severe OSAS group had a higher BMI than the control group, but the difference was not significant. Larger scale studies may better clarify this possible relationship between obesity, OSAS and periodontal diseases.

Salivary biomarkers may provide information for the assessment of the risk or the presence of periodontal disease [39]. Salivary IL-6 levels were significantly higher in the

two OSAS groups compared to the control group suggesting that OSAS might be able to increase the salivary level of this pro-inflammatory cytokine irrespective of the severity of OSAS. Novelty of the present IL-6 finding is the positive correlation between serum and salivary concentrations of this cytokine. The salivary IL-6 is likely to be derived from serum where it was 7-8 fold higher in concentration, but a contribution may also derive from inflammation of local periodontal tissues. Higher salivary IL-6 concentrations have been reported in patients with periodontitis [40] and further studies analysing GCF samples may shed more light on this hypothesis. It must be noted that it is still unclear whether there is a causal relationship between OSAS and the increased salivary IL-6.

The present findings revealed similar serum levels of IL-6, TNF- $\alpha$ , sRANKL, OPG, and apelin in individuals with or without OSAS. Serum sRANKL and OPG data in the OSAS patients are documented for the first time. However, previous studies demonstrated that the concentrations of IL-6 [12,41] and TNF- $\alpha$  [41] were higher in plasma samples of OSAS patients. The present findings did not appear to contradict the earlier study, since higher median levels of TNF- $\alpha$  and IL-6 were observed in our investigation but levels in OSAS groups were not statistically significantly different from the control group serum levels. This apparent discrepancy between TNF- $\alpha$  and IL-6 levels in OSAS in different investigations might be explained by possible differences in clinical periodontal status as well as demographic variables of the participants particularly as circulating levels of IL-6 [42] and TNF- $\alpha$  [43], which have been shown to be higher in patients with periodontitis. However, it is just as likely that there were not sufficient numbers in our investigation for the results to reach statistical significance.

Salivary apelin levels in OSAS and non-OSAS patients suggest OSAS increases salivary apelin. The highest apelin concentrations were found in saliva samples of the severe OSAS group. The mechanism resulting in higher salivary apelin levels in the

OSAS groups remain unclear, since there was no statistically significant difference in serum apelin concentration. Nevertheless, serum levels appeared to be higher in the OSAS groups. It might be questioned whether salivary apelin is derived entirely from the circulation. However, mechanisms leading to acquisition and accumulation of apelin in saliva remain to be elucidated. As salivary flow rate was not measured in the present study, no judgement can be made on this point. Apelin may be released by many cell types in the oral cavity in response to bacterial activity. There is also a possibility that some other factors in the saliva of OSAS patients are capable of interfering with the apelin assay, but this remains unknown. Apelin is a hormone with effects on cardiovascular system and glucose homeostasis [44]. It is expressed by human adipocytes and up regulated by insulin and obesity [45], as is IL-6 [46]. Recently, periodontitis has been suggested to contribute to adipose tissue inflammation by promoting insulin resistance [47]. Both OSAS [48] and periodontitis [47] are associated with insulin-resistance, and insulin-induced apelin expression has been demonstrated in adipocytes [49]. The presence and severity of periodontitis might influence salivary apelin and IL-6 originating from buccal adipocytes may be involved in this response. Further studies evaluating apelin levels in GCF of OSAS patients with and without periodontal disease in relation with insulin-resistance may help to clarify the possible relation between these two diseases.

Henley et al. [15] demonstrated significantly decreased plasma apelin levels after the CPAP therapy suggesting that various hormones such as apelin may play a role in OSAS. Zirlik et al. [50] also evaluated the plasma apelin levels in OSAS patients in comparison to healthy controls and reported similar plasma apelin levels in study groups. The present findings are in line with those of Zirlik et al. [50] as serum apelin levels were similar in the OSAS and non-OSAS patients. The present study is the first one to consider

the severity of OSAS in regards with serum apelin levels and suggested that OSAS severity does not appear to have a prominent effect on circulating apelin level but on saliva levels. Since many cell types including osteoblasts are known to secrete apelin and osteoblasts in particular proliferate in response to this molecule [51], there are important ramifications which have not been explored in this study particularly as osteoblasts are key players in bone metabolism.

The demographic variables and clinical periodontal parameters were well balanced in the present study groups. However, inclusion of both current and former smokers may be regarded as a limitation of the present study, as smoking can affect both salivary and serum cytokine levels [24]. On the other hand, the numbers of current/former smokers appeared to be balanced in the study groups and similar effects of smoking might be expected on the biochemical measurements in each study group.

## **Conclusion**

In conclusion, the present findings indicate that there is a marked shift in the presence of particular oral and periodontally relevant microorganisms in subgingival plaque. This finding suggests that OSAS has a bearing on the development of periodontal inflammation. A positive correlation between serum and salivary IL-6 concentrations in OSAS and the presence and severity of OSAS seem to have little effect on serum IL-6, TNF- $\alpha$ , OPG, sRANKL, apelin and salivary TNF- $\alpha$ , OPG, and sRANKL levels despite the finding that the number of apnea and duration of apnea correlated with PD and CAL. The increase in salivary concentrations of IL-6 and apelin may have an impact on or be due to the presence of periodontal disease in patients with OSAS. However, further studies with larger sample sizes, which include subgroups based on periodontal diagnosis, are required to establish a causal relationship between OSAS and periodontal diseases.

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**Conflict of Interest and Source of funding**

The authors declare that they have no conflict of interest. The study has been funded solely by the institutions of the authors.



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**Table 1:** Demographic variables and periodontal parameters of the patients. Values are given as mean  $\pm$  standard deviation.

	<b>Control</b>	<b>Mild-to-Moderate OSAS</b>	<b>Severe OSAS</b>
<b>Age</b> (years)	43.23 $\pm$ 9.08	49.88 $\pm$ 11.47	45.36 $\pm$ 9.81
<b>Gender</b> (F/M) (n)	8/5	8/9	4/18*
<b>Smoking</b> (Non-smoker/smoker/ex-smoker)	4/5/4	12/3/2	11/7/4
<b>Number of teeth present</b>	24.54 $\pm$ 3.23	25.29 $\pm$ 3.31	26.23 $\pm$ 2.29
<b>PD</b> (mm)	2.05 $\pm$ 0.45	2.41 $\pm$ 0.49	2.50 $\pm$ 0.79
<b>CAL</b> (mm)	2.22 $\pm$ 0.63	2.68 $\pm$ 0.72	3.04 $\pm$ 1.29
<b>PI</b>	1.44 $\pm$ 0.55	1.27 $\pm$ 0.55	1.61 $\pm$ 0.85
<b>Periodontal Status</b> Healthy/Gingivitis/ Periodontitis	6/6/1	4/8/5	8/6/8
<b>Sites (%) PD &gt; 4mm</b>	2.10 $\pm$ 5.05	7.02 $\pm$ 11.21	11.69 $\pm$ 13.96
<b>BOP</b> (%)	24.73 $\pm$ 17.63	33.11 $\pm$ 16.65	33.43 $\pm$ 24.50
<b>AHI</b> (events/h)	2.64 $\pm$ 1.82 <sup>†</sup>	17.24 $\pm$ 7.90 <sup>‡</sup>	67.49 $\pm$ 30.39
<b>BMI</b> (kg/m <sup>2</sup> )	31.71 $\pm$ 4.56	31.85 $\pm$ 5.32	34.18 $\pm$ 7.24

\* Statistical significant difference compared to control group (p=0.024).

<sup>†</sup> Statistical significant difference compared to OSAS groups (p<0.0001).

<sup>‡</sup> Statistical significant difference compared to control and severe OSAS groups (p<0.0001).

**Table 2:** Proportion of PCR positive individuals for each of the tested microorganisms.

Microbe	Control		mild/moderate OSAS		severe OSAS	
<i>A. actinomycetemcomitans</i>	7/13	54%	17/19	89%	16/22	73%
<i>A. naeslundii</i>	12/13	92%	19/19	100%	20/22	91%
<i>F. nucleatum</i>	11/13	85%	12/19	63%	15/22	68%
<i>P. gingivalis</i>	8/13	62%	15/19	79%	17/22	77%
<i>P. intermedia</i>	4/13	31%	3/19	16%	2/22	9%
<i>P. micra</i>	8/13	62%	13/19	68%	13/22	59%
<i>S. oralis</i>	11/13	85%	18/19	95%	18/22	82%
<i>S. mutans</i>	13/13	100%	19/19	100%	22/22	100%
<i>T. denticola</i>	5/13	38%	12/19	63%	13/22	59%
<i>T. forsythus</i>	8/13	62%	13/19	68%	15/22	68%
<i>V. dispar</i>	11/13	85%	17/19	89%	15/22	68%

**Table 3:** Salivary and serum cytokine levels in OSAS and non-OSAS patients (pg/mL).

Saliva	Control		Mild/Moderate		Severe	
	Median (q1 - q3)	Mean $\pm$ std	Median (q1 - q3)	Mean $\pm$ std	Median (q1 - q3)	Mean $\pm$ std
<b>TNF</b>	5.3(5.1 - 9.5)	6.8 $\pm$ 2.2	7.6(4.7 - 9.15)	10.6 $\pm$ 10.1	8.6(4.4 - 13.1)	10.2 $\pm$ 9.8
<b>sRANKL</b>	319.7(131.4 - 808.8)	482.8 $\pm$ 442.9	290.2(156.3 - 739.3)	466.3 $\pm$ 404.5	231.3(125.8 - 342.2)	465.7 $\pm$ 391.6
<b>OPG</b>	830.5(329.7 – 1057.0)	712.0 $\pm$ 376.9	709.1(536.0 - 829.7)	689.3 $\pm$ 255.8	785.6(614.1 - 955.5)	679.5 $\pm$ 250.9
<b>OPG/RANKL</b>	0.55(0.1 – 2.2)	1.5 $\pm$ 2.2	0.4(0.2 - 1.4)	1.3 $\pm$ 2.4	0.3(0.1 - 0.5)	1.3 $\pm$ 2.3
<b>IL-6</b>	8.8 (6.9 - 11.9)	8.5 $\pm$ 2.5	14.4(9.2 - 17.2)*	18.1 $\pm$ 13.1*	13.1(8.7 - 20.7)*	16.6 $\pm$ 12.3*
<b>Apelin</b>	20.3(9.9 - 141.4)	103.3 $\pm$ 204.8	35.3(6.5 - 245.8)	222.5 $\pm$ 387.2	223.1(20.6 - 877.2)*	210.2 $\pm$ 378.4*
Serum	Control		Mild/Moderate		Severe	
	Median (q1 - q3)	Mean $\pm$ std	Median (q1 - q3)	Mean $\pm$ std	Median (q1 - q3)	Mean $\pm$ std
<b>TNF</b>	91.2(58.7 - 97.1)	91.3 $\pm$ 64.6	95.1(74.5 - 106.4)	86.7 $\pm$ 40.5	69.2(30.5 - 128.4)	99.5 $\pm$ 64.63
<b>sRANKL</b>	607.1(319.0 - 718.8)	559.9 $\pm$ 296.6	514.9(207.0 - 754.5)	532.0 $\pm$ 371.1	635.7(276.6 - 678.9)	536.2 $\pm$ 358.9
<b>OPG</b>	1107.0(534.2 - 1308.0)	980.0 $\pm$ 475.9	1026(933.7 - 1488.0)	1195.0 $\pm$ 425.6	1121.0(802.9 - 1535.0)	1142.0 $\pm$ 461.6
<b>OPG/RANKL</b>	0.6(0.3 - 1.5)	1.0 $\pm$ 1.4	0.4(0.1 - 0.8)	0.6 $\pm$ 0.6	0.6(0.2 - 1.2)	0.7 $\pm$ 0.6
<b>IL-6</b>	80.4(59.8 – 104.7)	82.4 $\pm$ 27.2	101.5(92.9 - 115.7)	99.1 $\pm$ 42.5	84.9(36.7 - 151.3)	99.2 $\pm$ 41.1
<b>Apelin</b>	39.0(19.3 - 51.20)	37.7 $\pm$ 21.9	54.7(25.2 - 141.9)	69.2 $\pm$ 58.9	50.1(19.8 - 65.8)	65.5 $\pm$ 58.8

\* Significantly higher than the control group (p&lt;0.05).



**Table 4.** The correlations between sleep apnea parameters and clinical periodontal parameters.

Correlations	PD		CAL	
	Rho	P	Rho	P
No.of apnea	0.272	0.052	0.315	<b>0.023</b>
Mean apnea (sec)	0.386	<b>0.006</b>	0.416	<b>0.003</b>
Longest apnea (sec)	0.322	<b>0.022</b>	0.331	<b>0.019</b>
Duration of apnea-hypopnea (min)	0.362	<b>0.009</b>	0.403	<b>0.003</b>

## Legends:

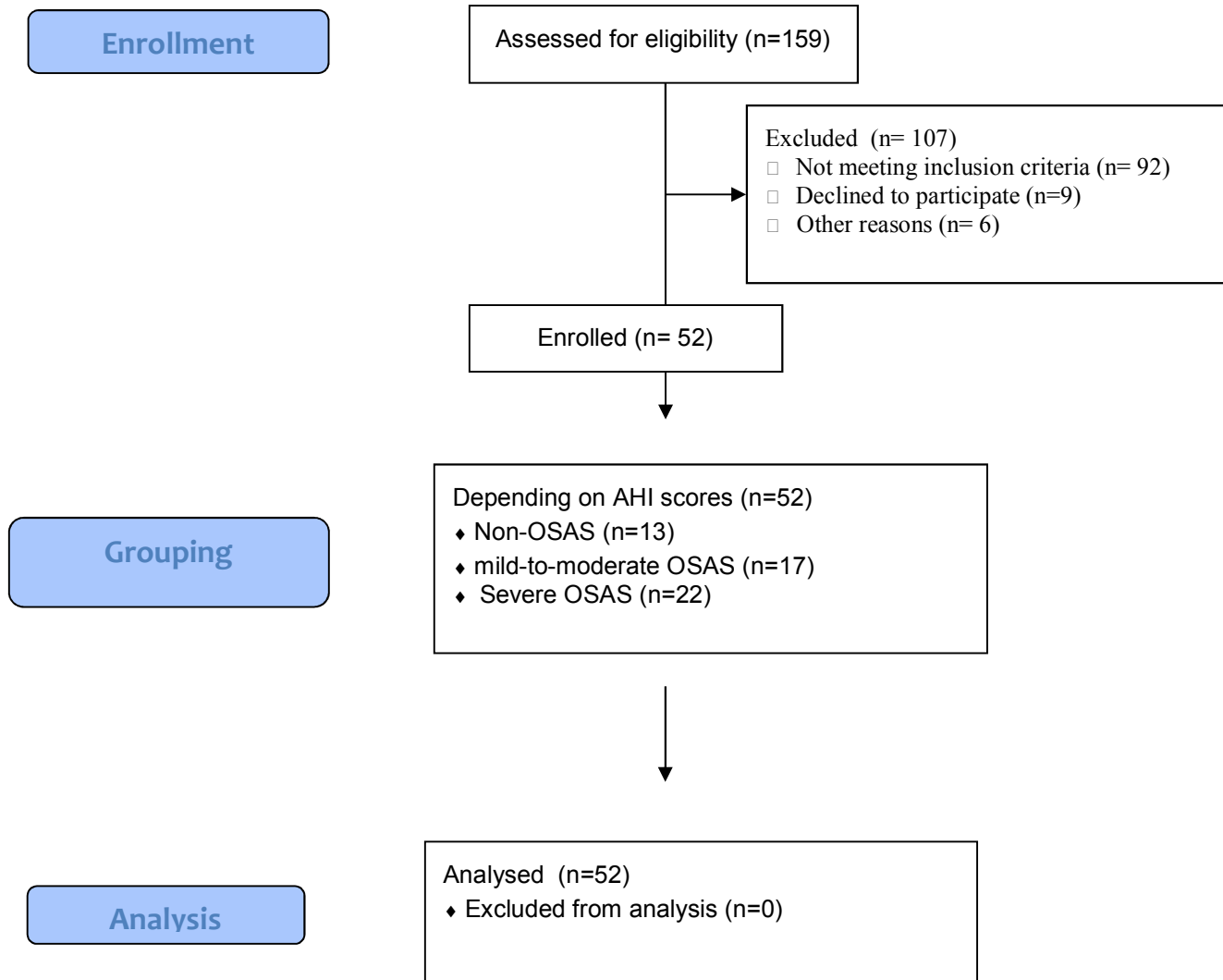
### Figure 1

**Figure 2.** Principal component analysis (PC) of *A. actinomycetemcomitans*, *A. naeslundii*, *F. nucleatum*, *P. gingivalis*, *P. intermedia*, *P. micra*, *S. oralis*, *S. mutans*, *T. denticola*, *T. forsythus* and *V. dispar* in sulcal and subgingival plaque of controls (C) without OSAS (circles), mild/moderate (m)OSAS (triangles) and severe (s)OSAS (squares) combined with varying levels of periodontal disease, gingivitis (+G) or periodontitis (+P) with increasing severity shown by increased intensity of grey in of the symbol. The chart shows a plot of the greatest variance in natural log transformations of bacterial counts plotted against the second greatest variance value of this data set. A shift in the composition of microbial plaque particularly in subjects with sOSAS and periodontal disease is indicated.

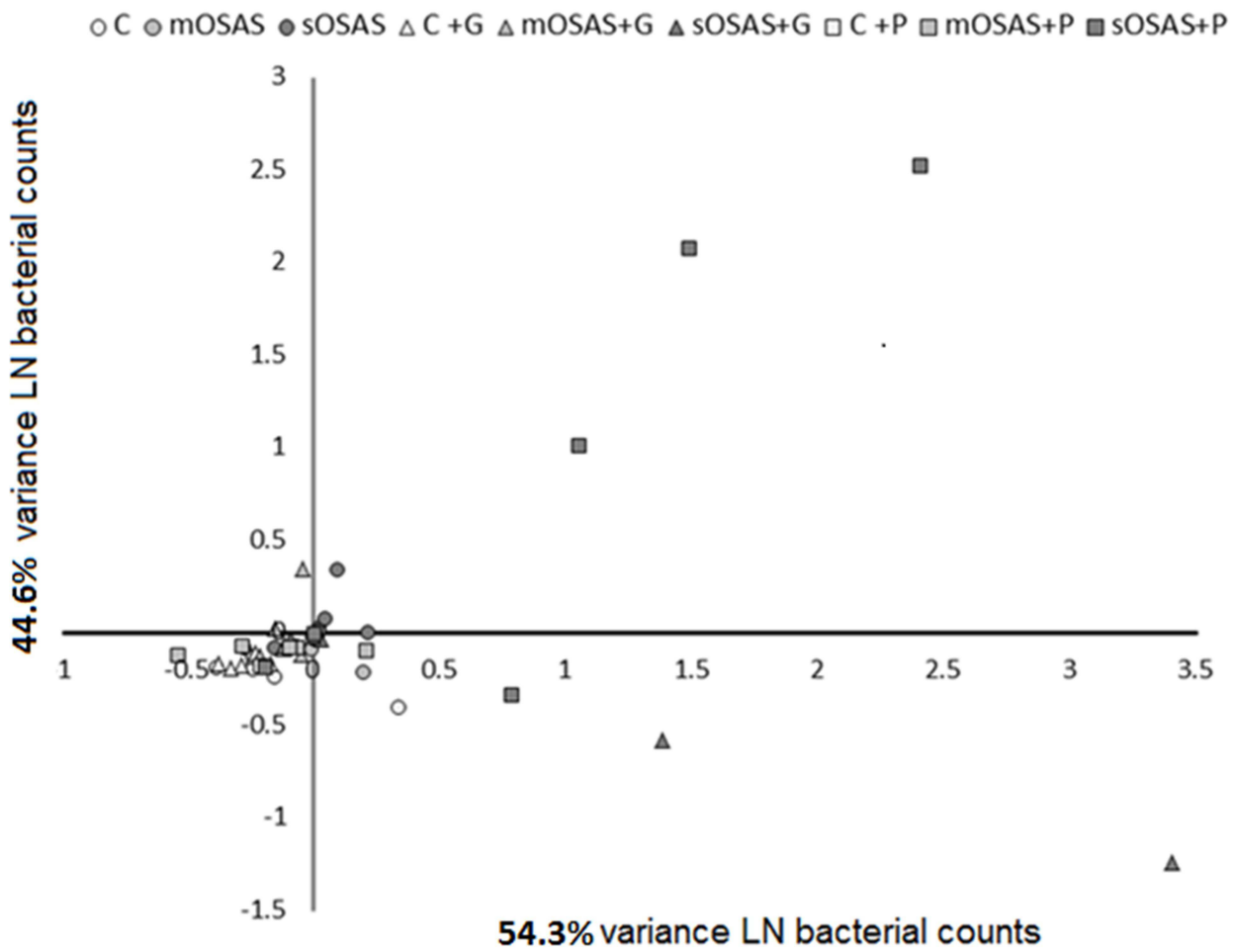
**Figure 3.** The stacked bar graphs indicate the cumulative numbers (A) and relative proportions of the sum (B) of *A. actinomycetemcomitans* (*A.actinom*), *A. naeslundii*, *F. nucleatum*, *P. gingivalis*, *P. intermedia*, *P. micra*, *S. oralis*, *S. mutans*, *T. denticola*, *T. forsythus* and *V. dispar* in sulcal and subgingival plaque of controls without OSAS, mild/moderate OSAS and severe OSAS.

\* Significant increase in microorganisms and in Gram negative microorganisms compared with the Control and Mild/moderate OSAS,  $p < 0.001$ .

**Figure 1. Flow Diagram**



**Figure 2:** Principal component analysis



Key:

C = Non-OSAS control group

mOSAS = mild/moderate OSAS

sOSAS= Severe OSAS

+G – with gingivitis

+P = with periodontitis

**Figure 3:** Detection of selected microorganisms in periodontal sulcal and subgingival plaque

